Developmental Cell
Article

Hdac1 and Hdac2 Act Redundantly to Control p63 and p53 Functions in Epidermal Progenitor Cells

Matthew LeBoeuf,[1,2] Anne Terrell,[1] Sohum Trivedi,[1] Satrajit Sinha,[5] Jonathan A. Epstein,[3] Eric N. Olson,[6] Edward E. Morrisey,[4] and Sarah E. Millar[1,*]
[1]Departments of Dermatology and Cell and Developmental Biology
[2]Cell and Molecular Biology Graduate Group
[3]Department of Cell and Developmental Biology and the Penn Cardiovascular Institute
[4]Cardiology Division, Department of Medicine
University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
[5]Department of Biochemistry, Center for Excellence in Bioinformatics and Life Sciences, State University of New York at Buffalo, Buffalo, NY 14203, USA
[6]Department of Molecular Biology, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
*Correspondence: millars@mail.med.upenn.edu
DOI 10.1016/j.devcel.2010.10.015

SUMMARY

Epidermal and hair follicle development from surface ectodermal progenitor cells requires coordinated changes in gene expression. Histone deacetylases alter gene expression programs through modification of chromatin and transcription factors. We find that deletion of ectodermal *Hdac1* and *Hdac2* results in dramatic failure of hair follicle specification and epidermal proliferation and stratification, phenocopying loss of the key ectodermal transcription factor p63. Although expression of p63 and its positively regulated basal cell targets is maintained in *Hdac1/2*-deficient ectoderm, targets of p63-mediated repression, including *p21*, *14-3-3σ*, and *p16/INK4a*, are ectopically expressed, and HDACs bind and are active at their promoter regions in normal undifferentiated keratinocytes. Mutant embryos display increased levels of acetylated p53, which opposes p63 functions, and p53 is required for HDAC inhibitor-mediated p21 expression in keratinocytes. Our data identify critical requirements for HDAC1/2 in epidermal development and indicate that HDAC1/2 directly mediate repressive functions of p63 and suppress p53 activity.

INTRODUCTION

The development of complex tissues from embryonic progenitor cells requires imposition of coordinated changes in gene expression programs. Conversely, development of malignancies involves broad suppression of differentiation programs and reversion to a more embryonic-like state. The accessibility and well-studied biology of the epidermis, and the prevalence of skin cancers, make it an ideal and important system in which to study these processes.

In mouse embryos, epidermal differentiation begins at approximately E8.5, when the single-layered ectoderm begins to express simple epithelial keratins KRT8 and 18, and the epidermal lineage-determining transcription factor p63 (Koster et al., 2007). Expression of basal cell keratins KRT5 and KRT14 is initiated at E9.5 (Byrne et al., 1994). Starting at E12.5, asymmetric cell divisions perpendicular to the plane of the epidermis are occasionally observed and are associated with the beginnings of epidermal stratification (Lechler and Fuchs, 2005). By birth the epidermis is composed of basal, suprabasal, granular and outer cornified layers, and forms a functional barrier that is essential for postnatal survival. Terminally differentiated cornified cells are continuously shed and replenished through the mitotic activity of basal progenitor cells that both self-renew and give rise to suprabasal progeny that enter the stratification program. Although most embryonic surface ectodermal cells undergo epidermal stratification, a subset of these cells interacts with the underlying mesenchyme to form a variety of ectodermal appendage organs (Mikkola and Millar, 2006).

p63 is essential for ectodermal appendage specification, epidermal proliferation and asymmetric cell division, and epidermal development, acting upstream of other early regulators of these processes (Koster, 2010; Laurikkala et al., 2006; Lechler and Fuchs, 2005; Mills et al., 1999; Truong and Khavari, 2007; Yang et al., 1999). Studies of p63 mutant thymus in vivo, and clonogenic assays in keratinocytes, support a key role for p63 in maintaining the proliferative potential of stem cells in stratified epithelia (Senoo et al., 2007).

Two classes of p63 isoforms are transcribed, TAp63 and ΔNp63, that respectively contain or lack an N-terminal transactivating domain. Each class includes multiple splice variants with differing activities, complicating mechanistic analysis (Blanpain and Fuchs, 2007). The predominant p63 isoform in embryonic epidermis, ΔNp63α, can function as either an activator or a repressor, depending on the target gene (McDade and McCance, 2010). ΔNp63α regulates the proliferation of epidermal progenitor cells by directly repressing expression of anti-proliferative target genes, such as *14-3-3σ* and *p21* (Westfall et al., 2003; Truong et al., 2006). ΔNp63α also directly represses

expression of the cell cycle regulators *p16/Ink4a* and *p19/Arf*, and defects in proliferation, epidermal stratification and ectodermal appendage development are partially rescued in *p63* mutants by concomitant loss of *p16/Ink4a* or, to a lesser extent, by loss of *p19/Arf* (Su et al., 2009). In addition to its repressive functions, ΔNp63α directly activates genes important for epidermal differentiation, including *Krt14*, *Ikkα*, *Fasn*, *Claudin1*, and *Gata3* (Chikh et al., 2007; Koster et al., 2007; Lopardo et al., 2008; Marinari et al., 2009; Romano et al., 2009). In vitro experiments suggest that p63 acts in opposition to the related transcription factor p53 in regulating epidermal proliferation but controls differentiation by a separate, p53-independent mechanism (Truong et al., 2006).

Lineage-determining transcription factors generally act in concert with chromatin regulators that permit access to sequence-specific binding sites and allow inheritance of gene expression programs (Kim et al., 1999). However, interactions of p63 with chromatin-modifying factors have yet to be described. Genetic analyses in the mouse, and experiments in organotypic culture, have revealed requirements for the histone methylase EZH2, and the DNA methyltransferase DNMT1, respectively, in maintaining epidermal progenitor cell proliferation (Ezhkova et al., 2009; Sen et al., 2010), but loss of these factors is associated with premature differentiation of basal cells, a phenotype distinct from that observed in *p63* null embryos.

Histone deacetylases (HDACs) remove histone acetylation marks, resulting in compaction of chromatin structure and transcriptional repression (Ruthenburg et al., 2007). HDACs operate by direct association with DNA-binding factors and by incorporation into large multifunctional repressor complexes such as Sin3, NuRD, coREST, and PRC2 (Brunmeir et al., 2009). In addition to functions in chromatin remodeling, HDACs deacetylate certain transcription factors, such as p53, resulting in their decreased activity (Higashitsuji et al., 2007; Tang et al., 2008). HDACs form a large family, of which class I HDACs, including the closely related proteins HDAC1 and HDAC2, show the strongest histone deacetylase activity (Haberland et al., 2009). Although HDAC1 and HDAC2 perform nonredundant roles in early development, these proteins coexist in repressive complexes, and tissue-specific deletion at later developmental stages reveals redundant functions for *Hdac1* and *Hdac2* in multiple different cell types (Brunmeir et al., 2009).

Several lines of evidence suggest important roles for histone deacetylation in epidermal development. Epidermal-specific deletion of *Mi-2β*, encoding an ATP-dependent chromatin-remodeling enzyme associated with HDAC1 and 2 in the NuRD complex, results in failure of hair follicle specification and gradual loss of basal epidermal progenitor cells (Kashiwagi et al., 2007). Class I and II HDACs also associate with the chromatin-remodeling ATPase BRG1, which is required for keratinocyte terminal differentiation (Indra et al., 2005). Treatment of adult skin with the HDAC inhibitor trichostatin A (TSA) causes hair follicle stem cells to proliferate and exit the stem cell compartment (Frye et al., 2007). However, the precise functions of specific HDAC isoforms in the epidermis in vivo have not been elucidated, and their requirements for embryonic epidermal development are unknown.

Here, we show that HDAC1 and HDAC2 are expressed in a dynamic and overlapping fashion in developing skin and hair follicles. Deletion of both genes in the epidermis results in a phenotype that strikingly resembles that of *p63* null skin, and derepression of negatively regulated ΔNp63 target genes, including the senescence factor *p16/Ink4a*. HDAC1/2 are present at the promoter regions of ΔNp63-repressed targets in keratinocytes, and histones in these regions are hyperacetylated following HDAC inhibition, indicating a direct requirement for HDAC1/2 in ΔNp63-mediated repression. In addition, p53 is hyperacetylated in *Hdac1/2* mutant epidermis, and experiments in keratinocytes indicate that increased p53 function contributes to the anti-proliferative effects of *Hdac1/2* deletion via induction of *p21* expression. These data reveal redundant and essential roles for *Hdac1/2* in controlling the activities of key regulators of epidermal development.

RESULTS

HDAC1 and HDAC2 Are Expressed in Dynamic and Overlapping Patterns in Developing Skin

Analysis of HDAC1 and HDAC2 expression in epidermal development revealed homogeneous expression of both proteins in epidermal nuclei at E13, prior to stratification of the epidermis (Figures 1A and 1B). At later developmental stages, HDAC1 and HDAC2 were expressed in all epidermal cells but localized most strongly to nuclei in outer, differentiating cell layers (Figures 1C–1J) and in the leading edges of developing hair follicles (Figures 1E and 1F).

Tissue-Specific Deletion of Either *Hdac1* or *Hdac2* Alone Does Not Impact Epidermal Development or Homeostasis

To delineate the functional requirements for *Hdac1* and *Hdac2* in epidermal development, we utilized *KRT14-Cre* transgenic mice in which Cre recombinase is efficiently expressed prior to hair follicle development or epidermal stratification (Liu et al., 2007), in combination with conditional loss of function alleles of either *Hdac1* or *Hdac2* (Montgomery et al., 2007). Consistent with the phenotypes of previously described tissue-specific *Hdac1* or *Hdac2* single mutants (Haberland et al., 2009), *KRT14-Cre* $Hdac1^{fl/fl}$ and *KRT14-*$Hdac2^{fl/fl}$ mice were viable and fertile and displayed no gross or histological skin abnormalities. Similarly, compound heterozygous, *KRT14-Cre* $Hdac1^{fl/fl}$ $Hdac2^{fl/+}$, and *KRT14-Cre* $Hdac1^{fl/+}$ $Hdac2^{fl/fl}$ mice showed no gross or histological defects in epidermal or hair follicle development or homeostasis (see Figure S1 available online; data not shown).

Hdac1/2 Epidermal Mutants Display Multiple, Severe Ectodermal Defects

To determine whether loss of both *Hdac1* and *Hdac2* resulted in epidermal abnormalities, we generated *KRT14-Cre* $Hdac1^{fl/fl}$ $Hdac2^{fl/fl}$ (DcKO) mice that lacked all four functional alleles in the epidermis. DcKO mice died perinatally with multiple and dramatic ectodermal defects (Figure 2). Immunostaining of DcKO-mutant skin revealed absence of both HDAC1 and HDAC2 proteins in surface epithelia by E14.5 (Figures 2A–2F). Consistent with key functions of HDAC1/2 in histone deacetylation, levels of histone H3 acetylated at lysine 9 (H3K9Ac) were markedly increased in E14.5 DcKO compared with control littermate epidermis (Figures 2G and 2H).

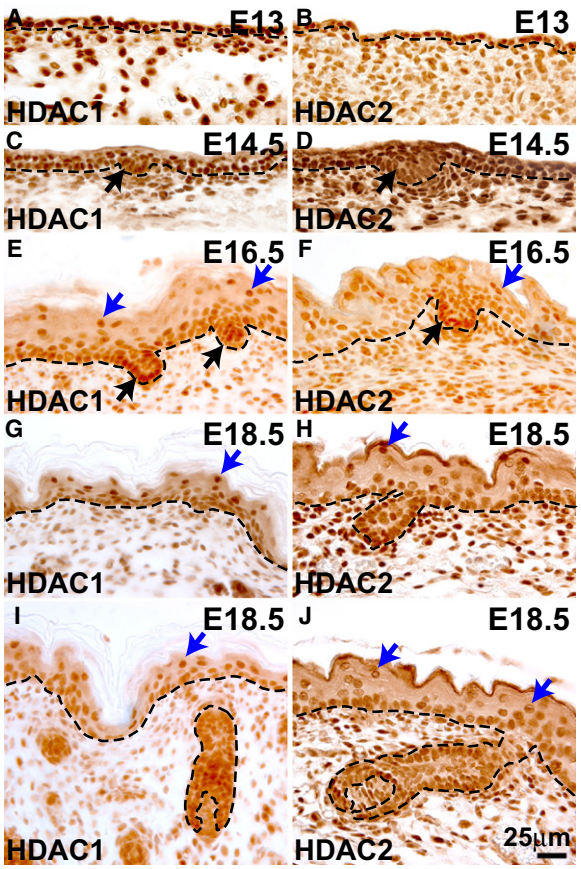

Figure 1. HDAC1 and HDAC2 Display Overlapping, Dynamic Expression in Developing Epidermis and Hair Follicles

Immunohistochemistry (brown) for HDAC1 and HDAC2 at the stages indicated.

(A and B) HDAC1 and HDAC2 are expressed in all cells of the E13 single-layer epidermis.

(C and D) HDAC1 and HDAC2 are ubiquitously expressed in E14.5 epidermis and hair follicle placodes (arrows).

(E and F) HDAC1 and HDAC2 are expressed most strongly at the leading edge of E16.5 hair follicles (black arrows) and in differentiating epidermal cells (blue arrows).

(G–J) At E18.5, HDAC1 and HDAC2 are expressed strongly in differentiating epidermal cells (blue arrows). Dashed-black lines indicate dermal-epidermal junctions. Scale bar in (J) applies to all images.

DcKO embryos displayed thin, smooth skin, failure of eyelid fusion, and failure of limb-digit separation (Figures 2I and 2J). Histological analysis showed that, instead of stratifying, the epidermis remained as a single layer throughout embryogenesis and lacked any signs of hair follicle development (Figures 2K–2P). Tooth development was initiated, consistent with the early timing of this process relative to *KRT14-Cre* activity (Liu et al., 2008); however, dental structures were abnormal in DcKO mutants at E16.5 (Figures 2Q and 2R) and degraded by E18.5 (Figures 2S and 2T). Formation of keratinized filiform papillae in tongue epithelium was absent in DcKO mutants, and like the epidermis, tongue surface ectoderm remained as a single layer throughout embryogenesis (Figures 2U and 2V). Histological analysis of DcKO embryonic limbs revealed failure of epidermal differentiation between the digits that is required for digit separation (e.g., Ingraham et al., 2006) (Figures 2W and 2X), and analysis of mutant eyes at E18.5 showed that eyelid development was arrested (Figure S2).

Hdac1/2 Are Required for Suprabasal Epidermal Differentiation and Initiation of Hair Follicle Development

To dissect the molecular basis for the abnormalities observed in DcKO embryos, we first examined the expression of molecular markers for epidermal stratification. The basal cell keratin, KRT14, was expressed similarly in control and DcKO epidermis at E14.5. At later embryonic stages, KRT14 was confined to basal cells in controls and remained expressed in the ectoderm of DcKO embryos. By contrast, KRT10, which marks suprabasal cells in control embryos from approximately E15, was not expressed in DcKO epidermis at any stage. Loricrin, a marker for the granular layer, was readily detected in control embryos at E16.5 but was not expressed at any stage in DcKO embryos (Figure 3A). To determine whether epidermal cells revert to a less-differentiated state in DcKO mutants, we asked whether the simple epithelial keratin, KRT18, is ectopically expressed. Immunostaining at E16.5 revealed absence of KRT18 expression in both control and DcKO epidermis (Figures S3A and S3B). Thus, basal epidermal development is initiated and maintained in DcKO embryos, but differentiation and stratification of the epidermis completely fail.

Like epidermal stratification, initiation of hair follicle development requires a global switch in the program of ectodermal differentiation. To determine the stage at which hair follicle development is blocked in DcKO mutants, we carried out whole mount in situ hybridization for *Ctnnb1* (encoding β-catenin), a very early marker for hair follicle placode initiation (Zhang et al., 2009). Patterned expression of *Ctnnb1*, corresponding to hair follicle pre-placodes, was observed in control epidermis at E14.0 but was absent in DcKO mutants, indicating that placode development was not initiated (Figure 3B). Similarly, nuclear β-catenin protein and its transcriptional partner LEF1 were elevated in control hair follicle placodes and downregulated in intervening epidermis at E14.5 (Jamora et al., 2003; Zhang et al., 2009), whereas DcKO epidermis displayed uniform, low-level expression of nuclear β-catenin and LEF1 (Figure 3C). Vibrissa and mammary placode formation is initiated prior to efficient expression of *KRT14-Cre* (Liu et al., 2007). Elevated *Ctnnb1* expression marked developing vibrissae and mammary buds in DcKO as well as control embryos (Figures S3C and S3D). These observations indicate that *Hdac1/2* are not required to maintain elevated *Ctnnb1* expression but, rather, are necessary to allow hair follicle fate acquisition. Thus, *Hdac1/2* are required for at least two different types of developmental transition in embryonic surface ectoderm: stratification and hair follicle initiation.

DcKO Embryos Display Progressive Defects in Proliferation and Apoptosis

To determine whether proliferation was affected by *Hdac1/2* deletion, we analyzed the percentage of phospho-histone H3 (PH3)-positive basal cells in mutant and control littermate epidermis. At E14.5, proliferation was decreased in DcKO

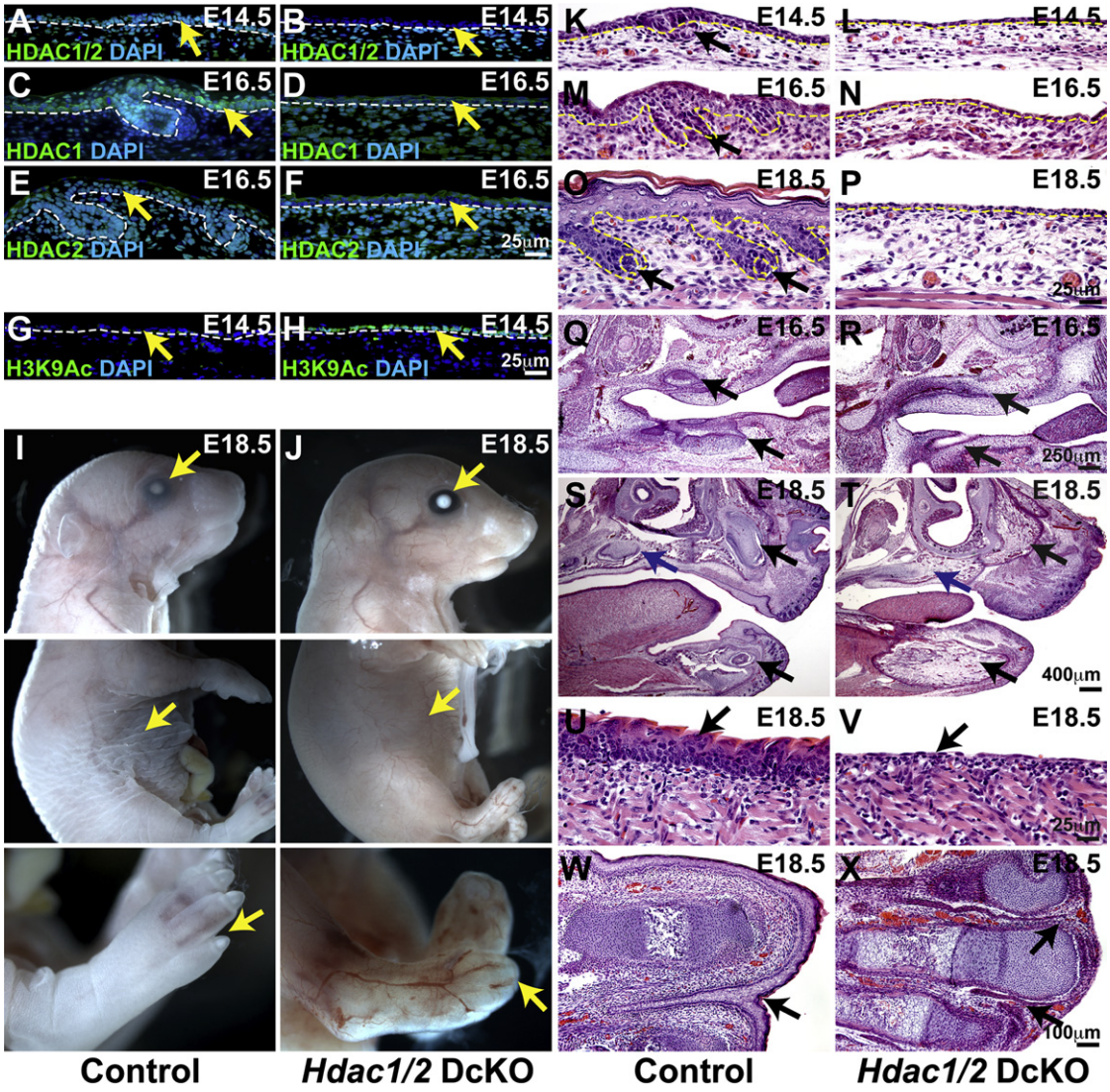

Figure 2. Embryos Lacking Epidermal HDAC1 and HDAC2 Display Striking Defects in Epidermal and Ectodermal Appendage Development

(A–F) Immunofluorescence (green) reveals expression of HDAC1 and HDAC2 in the epidermis and developing hair follicles of control E14.5 and E16.5 *Hdac1*$^{fl/fl}$ *Hdac2*$^{fl/fl}$ littermate skin (A, C, and E) and their absence in *KRT14-Cre Hdac1*$^{fl/fl}$ *Hdac2*$^{fl/fl}$ (DcKO) epidermis (B, D, and F) (arrows).

(G and H) Immunofluorescence (green) reveals increased levels of H3K9Ac in DcKO (H) compared with control (G) epidermis (arrows).

(I and J) Open eyes, thin smooth skin, and failure of digit septation in a DcKO embryo at E18.5 (J) compared with a control *KRT14-Cre Hdac*$^{fl/+}$ *Hdac2*$^{fl/fl}$ littermate (I) (arrows). (K–X) Hematoxylin and eosin staining reveals defects in stratification and ectodermal appendage development in DcKO compared with control littermate embryos.

(K and L) Absence of hair follicle placodes in E14.5 DcKO epidermis (L) compared with control skin (K, arrow).

(M–P) DcKO epidermis remains as a single-cell layer at E16.5 (M and N) and E18.5 (O and P) instead of stratifying, and lacks hair follicles.

(Q–T) Abnormal DcKO dental lamina morphology at E16.5 (Q and R, arrows) and degeneration of incisor (black arrows) and molar (blue arrows) dental structures at E18.5 (S and T).

(U and V) Failure of filiform papilla morphogenesis in E18.5 DcKO tongue surface ectoderm (arrows).

(W and X) Sections through E18.5 hindlimbs reveal digit formation and failure of epidermal differentiation between the digits in the DcKO (arrows).

Nuclei in (A)–(H) are DAPI stained (blue). Dashed lines mark dermal-epidermal borders. Scale bar in (F) applies to (A)–(F), scale bar in (H) applies to (G) and (H), scale bar in (P) applies to (K)–(P), and scale bars in (R), (T), (V), and (X) also apply to their respective littermate controls.

See also Figures S1 and S2.

mutants to approximately 56% of the levels seen in controls (20 fields of view counted at 40× magnification for each genotype; $p < 1 \times 10^{-4}$). By E16.5 this percentage had decreased to only 17% of control levels ($p < 1 \times 10^{-5}$) (Figures 3D and 3F; see also Figures S3E–S3H). The progressive nature of this proliferation defect is consistent with failure of self-renewal of progenitor cells (Sen et al., 2010; Senoo et al., 2007).

To determine whether programmed cell death contributed to the *Hdac1/2* mutant phenotype, we utilized a TUNEL assay. Interestingly, there was no statistically significant difference in

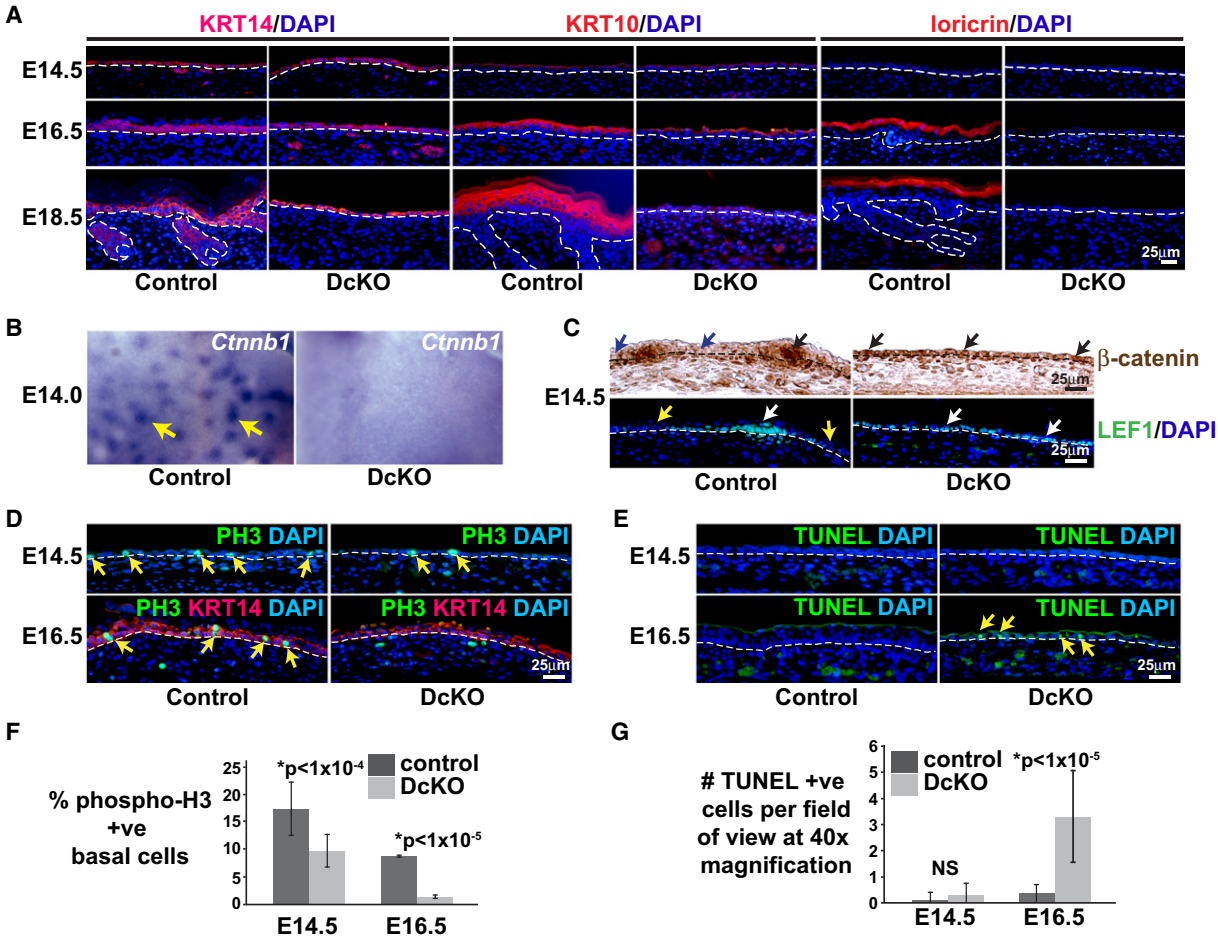

Figure 3. Loss of Epidermal *Hdac1/2* Causes Failure of Epidermal Stratification, Absence of Hair Follicle Specification, and Progressive Defects in Proliferation and Cell Survival

(A) Immunofluorescence (red) with antibodies to the basal layer marker KRT14, the suprabasal marker KRT10, and the granular layer marker loricrin reveals expression of KRT14 but lack of KRT10 and loricrin in DcKO epidermis at E14.5–E18.5. The weak signal in E16.5 DcKO skin incubated with anti-KRT10 is due to background fluorescence in the periderm.

(B) Whole mount in situ hybridization (blue-purple) for *Ctnnb1* reveals formation of hair follicle pre-placodes in E14.0 control littermate skin (arrows) and their absence in DcKO skin.

(C) Immunohistochemistry for β-catenin (brown) and immunofluorescence for LEF1 (green) reveal upregulated expression in developing hair follicle placodes (black or white arrows) and lower or absent expression in inter-placodal epidermis (blue or yellow arrows) in controls, and uniform low-level expression in DcKO epidermis (black or white arrows).

(D) Immunofluorescence for PH3 (green) reveals decreased proliferation in DcKO epidermis at E14.5 and almost absent proliferation at E16.5 (arrows). E16.5 samples were costained with anti-KRT14 (red) to mark basal cells.

(E) TUNEL staining (green) reveals increased cell death in DcKO embryos at E16.5 (arrows) but not at E14.5 compared to littermate controls.

(F) Quantification of the percentage of PH3-positive basal layer cells reveals a statistically significant decrease in proliferation in DcKO compared with control embryos at E14.5 and a further decrease in the relative rate of proliferation at E16.5. Twenty fields of view were counted for each genotype.

(G) Quantification of the number of TUNEL-positive cells per field of view at 40× magnification reveals a statistically significant increase in the number of apoptotic basal cells in DcKO embryos at E16.5, but not at E14.5. Twenty fields of view were counted for each genotype.

Statistical data are represented as mean values; error bars indicate SEM. Dashed lines indicate dermal-epidermal junctions. NS, not statistically significant. See also Figure S3.

the rate of programmed cell death between DcKO and control epidermis at E14.5 (20 fields of view counted at 40× magnification for each genotype; $p = 0.32$), indicating that the initial defects observed in DcKO skin were not due to increased apoptosis. However, by E16.5 an approximately 6-fold increase in the rate of basal cell apoptosis was observed in DcKO compared with control epidermis ($p < 0.4 \times 10^{-5}$) (Figures 3E and 3G).

Hdac1/2 Are Not Required for Maintaining Expression of ΔNp63 and Its Positively Regulated Basal Cell Targets

The ectodermal defects observed in DcKO embryos were strikingly reminiscent of many of the phenotypes described in embryos lacking *p63*, including lack of epidermal stratification, decreased proliferation, failure of hair follicle specification, defective eyelid development, absence of tongue filiform

papillae, and defective dental development (Laurikkala et al., 2006; Lechler and Fuchs, 2005; Mills et al., 1999; Yang et al., 1999). Of particular note, similar to *p63* null skin, *Hdac1/2* deficient embryonic epidermis exhibited failure of proliferation in the absence of premature differentiation.

Therefore, we asked whether expression of p63 is maintained following deletion of *Hdac1/2*. Immunostaining using an antibody that recognizes all p63 isoforms revealed expression of p63 in basal cells and in some immediately suprabasal cells in control embryos at E14.5–E18.5. In DcKO embryos, strong expression of p63 was observed in the epidermis at E14.5 and was maintained at E16.5 and E18.5 (Figure 4A). To determine whether similar p63 isoforms were expressed in control and DcKO skin, we used antibodies specific for ΔNp63 and TAp63 isoform classes (Romano et al., 2009). ΔNp63 isoforms predominated in both control and DcKO epidermis at E14.5 (Figures 4B and 4C). As previously reported, TAp63 isoforms were not detected in control skin at this stage (Laurikkala et al., 2006; Romano et al., 2009) and were also not detectable in the mutant (Figures 4D and 4E).

Because p63 isoform expression appeared unchanged by deletion of *Hdac1/2*, we asked whether *Hdac1/2* might mediate p63 function. Positively regulated targets of ΔNp63 in epidermal basal cells include *Krt14* (Romano et al., 2009), fatty acid synthase (*Fasn*) (Lefkimmiatis et al., 2009), the cell cycle progression regulator *Cdc25c* (Lefkimmiatis et al., 2009), *claudin1* (Lopardo et al., 2008), and *Ikkα* (Candi et al., 2006; Marinari et al., 2009). As shown in Figure 3A, maintenance of KRT14 expression was unaffected by *Hdac1/2* deletion. Similarly, FASN, CDC25, Claudin1, and IKKα were expressed in DcKO epidermis (Figures 4F–4K; Figures S4A–S4D). Consistent with absence of stratification, GATA3, a positively regulated target of TAp63 in suprabasal cells (Chikh et al., 2007), was not expressed in DcKO epidermis (Figures S4E and S4F).

ΔNp63-Repressed Targets Are Upregulated in *Hdac1/2*-Deficient Epidermis

Because expression of positively regulated basal cell ΔNp63 targets was unaffected by *Hdac1/2* deletion, we next examined expression of 14-3-3σ and p21, which are direct targets of ΔNp63-mediated repression (Westfall et al., 2003). In control epidermis at E14.5, 14-3-3σ was expressed in emerging suprabasal layers but was largely absent from the basal layer. However, E14.5 DcKO embryos displayed weak ectopic expression of 14-3-3σ in the basal layer (Figures 4L and 4M). This difference was more pronounced at E16.5, when 14-3-3σ was strongly expressed in suprabasal cells but absent from the basal layer in controls, and DcKO mutants displayed strong ectopic 14-3-3σ expression in basal cells (Figures 4N and 4O). Similarly, p21 was weakly expressed in control basal cells at E14.5 and E16.5 and displayed increased immunoreactivity in mutant epidermis (Figures 4P–4S).

The *Ink4a/Arf* locus produces two independent proteins: p16/INK4a, which specifically binds and inhibits the cyclin-dependent kinases 4 and 6, preventing phosphorylation of the retinoblastoma tumor suppressor protein; and p19/ARF, which stabilizes p53. p63 has been reported to directly repress both *p16/Ink4a* and *p19/Arf* (Su et al., 2009). However, ectodermal defects in *p63* null embryos, including proliferation, stratification, and hair follicle specification, are rescued to a greater extent by concomitant deletion of *p16/Ink4a* than by loss of *p19/Arf* (Su et al., 2009). Interestingly, expression of p19/ARF appeared unaffected by *Hdac1/2* mutation (Figures S4G–S4J). However, p16/INK4a was dramatically overexpressed in DcKO embryos at both E14.5 and E16.5 (Figures 4T–4W). These data indicate that key repressive functions of ΔNp63, including suppression of *p16/Ink4a*, require HDAC1/2.

To determine whether the requirement for HDAC1/2 in ΔNp63-mediated repression is direct, we first asked whether HDAC1/2 are present at the promoters of the *14-3-3σ*, *p21* and *p16/INK4a* genes in undifferentiated primary human epidermal keratinocytes (HEKs). Chromatin immunoprecipitation (ChIP) assays with antibodies to HDAC1 and HDAC2, and PCR primers that amplify promoter regions containing p63-binding sites for each of these genes, revealed specific binding of both HDAC1 and HDAC2 in each case (Figure 4X, left panels). We next wanted to determine whether HDACs are required for histone acetylation at these promoter regions. To address this question we carried out ChIP assays using the same primers to amplify *14-3-3σ*, *p21* and *p16/INK4a* promoter sequences, antibodies to H3K9Ac, and extracts of keratinocytes that had been cultured with or without the HDAC inhibitor TSA. These experiments revealed increased histone acetylation at the *14-3-3σ*, *p21* and *p16/INK4a* promoters in TSA-treated compared with control-treated cells (Figure 4X, right panels). Quantification of bound H3K9Ac levels by real-time PCR coupled with ChIP revealed that the increases in H3K9Ac at the *p21*, *14-3-3σ*, and *p16INK4a* promoter regions were statistically significant ($p < 0.05$ for all TSA-treated samples compared with control treated, except for *14-3-3σ* in samples treated with TSA for 6 hr, for which $p = 0.05$) (Figure S4K). By contrast, levels of acetylated histone H3 at the negative control *GAPDH* promoter were not significantly increased in TSA-treated compared with control-treated cells (Figure S4K). Thus, HDACs are specifically active at ΔNp63-repressed promoters. These data are consistent with a direct requirement for HDAC1/2 in ΔNp63-mediated repression of cell cycle inhibitory genes and suggest that proliferative, stratification, and ectodermal appendage defects in *Hdac1/2* null epidermis are due at least in part to failure of ΔNp63-repressive functions.

HDAC1/2 Suppress P53 Hyperacetylation in Embryonic Epidermis

P53 positively regulates several targets of p63 repression, including *14-3-3σ* and *p21* (el-Deiry et al., 1993; Hermeking et al., 1997), which are ectopically or overexpressed in DcKO epidermis. In addition, levels of PCNA, which is indirectly suppressed by P53 (Saifudeen et al., 2002), and a P53-activated direct target GADD45A (Rosemary Siafakas and Richardson, 2009), were respectively decreased and increased in DcKO compared with control epidermis at E16.5 (Figures 5A–5H). In vitro studies suggest that P53 activity is repressed in an HDAC1-dependent manner through removal of acetylation marks from its C-terminal lysine residues (Higashitsuji et al., 2007; Ito et al., 2002; Tang et al., 2008). Therefore, we asked whether acetylated P53 levels were increased in DcKO epidermis in vivo. Immunofluorescence revealed marked increases in the levels of p53 acetylated at either lysine 379 or lysine 386 in DcKO compared with control littermate epidermis

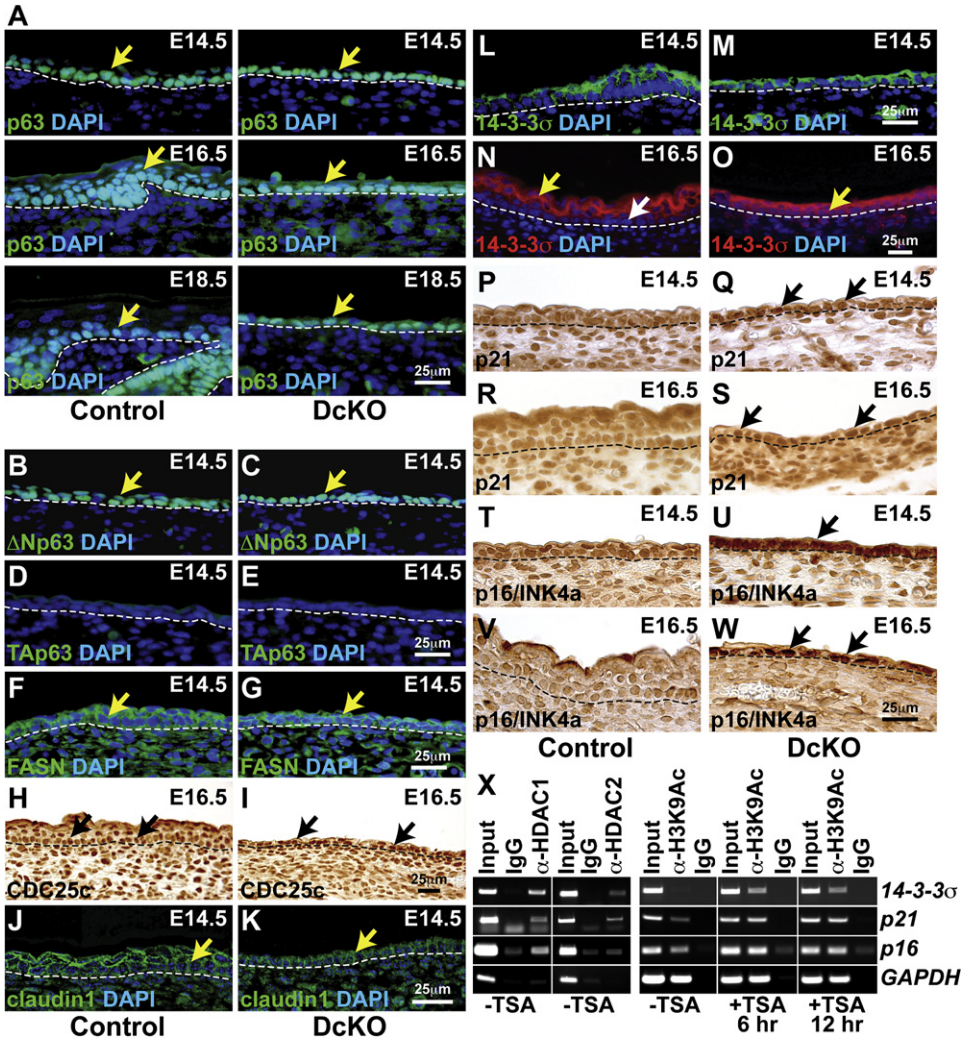

Figure 4. *Hdac1* and *Hdac2* Are Required for Repression of Negatively Regulated ΔNP63 Target Genes

(A) Immunofluorescence with pan-p63 antibody (green) reveals expression in the basal layer of littermate controls and in the single-layered DcKO epidermis at E14.5, E16.5, and E18.5 (arrows).

(B–E) Expression of ΔNP63 (B and C, arrows) and undetectable expression of TAP63 (D and E) in both control (B and D) and DcKO (C and E) epidermis at E14.5.

(F–K) Positively regulated p63 targets FASN (F and G) (green), CDC25c (H and I) (brown), and claudin1 (J and K) (green) are similarly expressed in single-layered DcKO (G, I, and K) and basal control (F, H, and J) epidermis (arrows).

(L–O) Immunofluorescence for 14-3-3σ at E14.5 (L and M) (green signal) and E16.5 (N and O) (red signal) reveals its expression in control suprabasal cells (N, yellow arrow) and absence in control basal cells (N, white arrow). In DcKO skin, 14-3-3σ is expressed in the single-layered epithelium at both E14.5 (M) and E16.5 (O) (yellow arrow).

(P–S) p21 immunostaining (brown) is slightly more intense in DcKO (Q and S) compared with control basal epidermis (P and R) at both E14.5 (P and Q) and E16.5 (R and S) (arrows).

(T–W) p16/INK4a (brown immunostaining) is dramatically upregulated in DcKO (U and W) compared with control (T and V) epidermis at both E14.5 (T and U) and E16.5 (V and W) (arrows). Dashed lines indicate dermal-epidermal boundaries.

(X) CHiP assays with extracts of untreated HEKs and HEKs treated with TSA for 6 or 12 hr, and antibodies to HDAC1, HDAC2, H3K9Ac, or rabbit IgG negative control, as indicated. Primers amplifying sequences that encompass *GAPDH*-negative control promoter regions or p63-binding sites in the promoter regions of the *14-3-3σ*, *p21*, and *p16/INK4a* genes were used for PCR as indicated.

All panels in (A) were photographed at the same magnification; scale bar in (E) applies to (B)–(E); scale bars in (G), (I), (K), (M), and(O) also apply to their respective littermate controls; and scale bar in (W) applies to (P)–(W).

See also Figure S4 and Table S1.

at both E14.5 and E16.5 (Figures 5I–5P). Thus, *Hdac1/2* are necessary to suppress hyper-acetylation of P53 in embryonic epidermis, and lack of this activity may contribute to upregulation of cell cycle inhibitory genes and the observed proliferative defects and apoptosis in DcKO embryos.

p53 Is Required for Upregulation of p21 in Keratinocytes in Response to HDAC Inhibition

To test more directly whether increased levels of acetylated p53 might be required for the observed upregulation of epidermal p21, we inhibited HDAC function in HEKs by treatment with

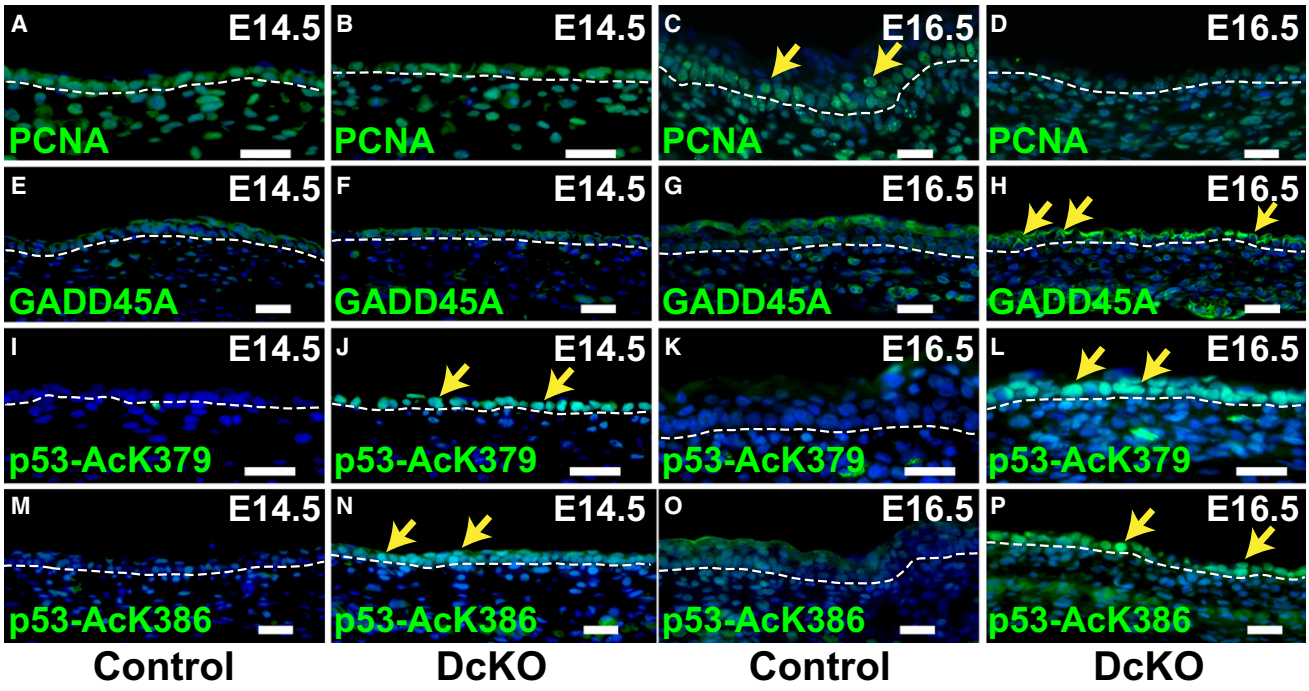

Figure 5. HDAC1/2 Suppress Hyper-Acetylation of p53 in Embryonic Epidermis

(A–D) PCNA (green) is expressed in control basal cell and some suprabasal cell nuclei at E14.5 (A) and E16.5 (C, arrows). PCNA is expressed in DcKO epidermis at E14.5 (B) but is downregulated by E16.5 (D).

(E–H) GADD45A (green) is expressed in control suprabasal, but not basal cells at E14.5 (E) and E16.5 (G). In single-layered DcKO epidermis, GADD45A is weakly expressed at E14.5 (F) and gives a strong signal at E16.5 (H, arrows).

(I–P) p53-AcK379 (I–L) and p53-AcK386 (M–P) (green) are undetectable in control basal nuclei at E14.5 (I and M) or E16.5 (K and O) and are weakly detected in a subset of differentiating nuclei at E16.5 (K and O). DcKO epidermis displays strong staining for acetylated p53-AcK379 (J and L) and p53-AcK386 (N and P) at both E14.5 (J and N) and E16.5 (L and P) (arrows). Dashed lines indicate dermal-epidermal junctions. Scale bars represent 25 μm.

TSA. Twenty-four hours of TSA treatment resulted in a statistically significant decrease in proliferation (Figures 6A, 6D, and 6G; Figure S5C) as well as dramatic increases in the intensity of immunofluorescence for acetylated p53 and for p21 (Figures 6B, 6C, 6E, and 6F). shRNA-mediated knockdown of *p53* or *p21* expression resulted in reduced immunostaining for the corresponding protein (Figures 6H, 6I, 6K, and 6L), decreased transcript levels to approximately 25% of those observed in control knockdown cells (Figures S5A and S5B), and inhibition of the anti-proliferative effects of TSA (Figures 6G; Figure S5C). Knockdown of *p21* in TSA-treated cells did not affect total p53 levels (Figures 6H and 6J). By contrast, knockdown of *p53* prevented accumulation of p21 protein and mRNA in response to TSA treatment (Figures 6K, 6M, and 6N). Thus, p53 is required for elevated p21 levels in response to HDAC inhibition in keratinocytes.

These data are consistent with a model in which increased levels of p53 activity contribute to proliferative defects in *Hdac1/2*-deficient epidermis. However, the relatively mild effects of loss of *Hdac1/2* on expression of p21 in DcKO mutants, compared with dramatic upregulation of the p53-independent gene *p16/Ink4a* by E14.5 (Figures 4P–4W), indicate that p53-independent loss of ΔNP63 repressor functions plays a major role. Although deletion of HDAC1/2 should immediately relieve ΔNp63-mediated repression of *p16/Ink4a*, accumulation of critical levels of acetylated p53 is likely required for increased p53 activity and p53-mediated target gene activation. This less direct mechanism (Figure 6O) may account in part for our observation that activation of some p53 targets is delayed relative to the increase in p16/INK4a expression. Because p53 is well established as a pro-apoptotic protein, this indirect mechanism may also explain the relatively late increase in apoptosis observed in *Hdac1/2* epidermal mutants (Figures 3E and 3G).

DISCUSSION

Here, we show that *Hdac1/2* perform essential functions in ectodermal appendage and epidermal development and are required for basal epidermal cells to acquire suprabasal or hair follicle placode fates. Consistent with specific defects resulting from *Hdac1/2* deletion in other tissues, *KRT14-Cre*-mediated loss of *Hdac1/2* produced an epidermal phenotype strikingly similar to that of embryos lacking the epidermal master regulator, p63. *Krt14* is a direct, positively regulated p63 target (Romano et al., 2009), and *KRT14-Cre*-mediated deletion of *Hdac1/2* occurs after initiation of *p63* expression, but prior to development of a suprabasal epidermal layer or specification of hair follicles (Liu et al., 2007). In line with this, both p63 and KRT14 were expressed in *Hdac1/2* epidermal mutants, and defects in morphogenesis of the limbs and ectodermal appendages such as mammary glands, vibrissae, and teeth that develop before onset of high levels of *KRT14-Cre* expression were less severe than the epidermal stratification and hair follicle phenotypes.

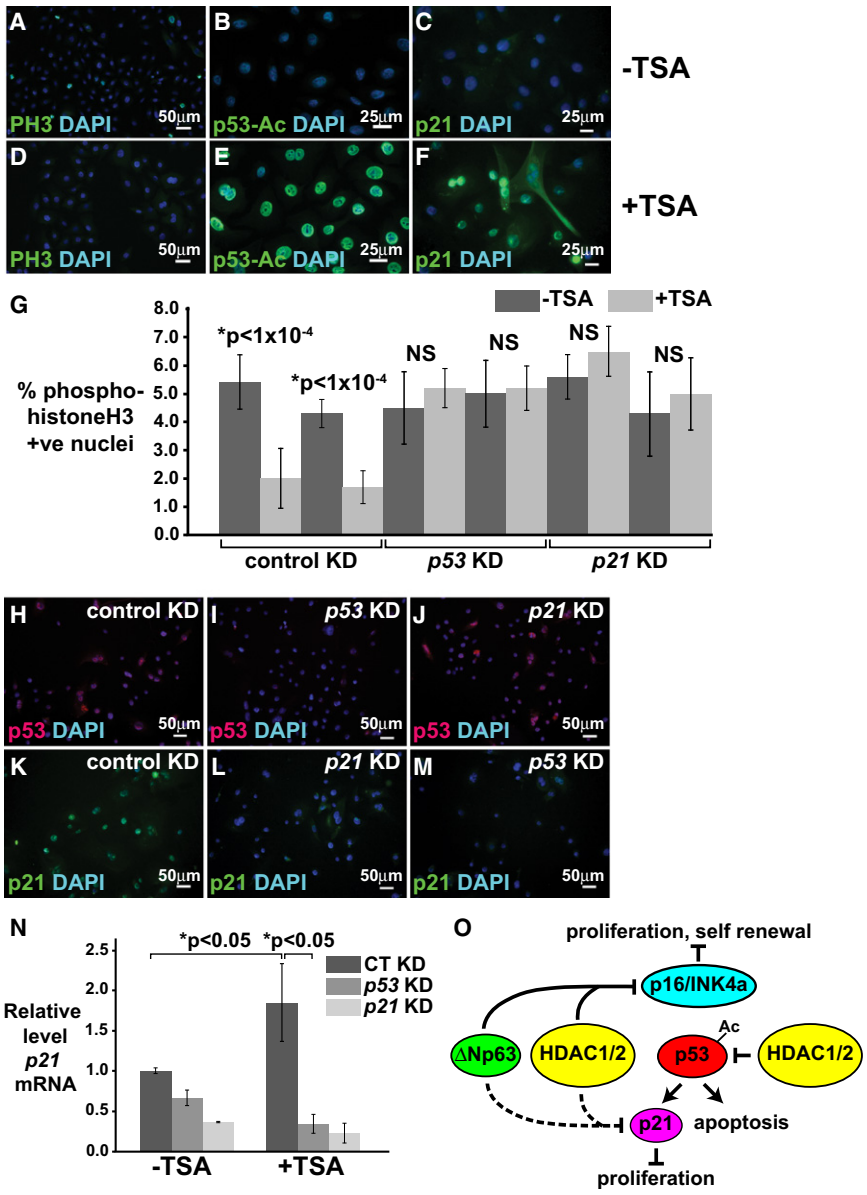

Figure 6. p53 Is Required for Elevated p21 Expression in Response to HDAC Inhibition in Keratinocytes

(A–F) TSA treatment of HEKs causes decreased proliferation and increased expression of acetylated p53 and p21. HEKs treated with 500 nM TSA in DMSO (D–F) or DMSO alone (A–C) were subjected to immunofluorescence (green) using antibodies to PH3 (A and D), p53-AcK379 (B and E), or p21 (C and F).

(G) The TSA-induced decrease in proliferation requires *p53* and *p21*. HEKs were transduced with retroviruses carrying control knockdown (KD), *p53* KD, or *p21* KD shRNAmir constructs as indicated and treated with 50 nm TSA in DMSO (light-gray bars) or DMSO only (dark-gray bars). The percentage of PH3-positive nuclei is indicated for each condition. Ten fields of view were counted per sample at 40× magnification, and the experiment was performed in duplicate.

(H–N) *p53* is required for the TSA-induced increase in p21 expression. HEKs were transduced with retroviruses carrying control (H and K), *p53* (I and M), or *p21* (J and L) knockdown shRNAmirs and treated with 50 nM TSA. Immunofluorescence with antibodies to total p53 (H–J) (red) or p21 (K–M) (green) reveals efficient knockdown of *p53* or *p21* with the corresponding shRNAmir (H, I, K, and L), and decreased p21 expression in *p53* knockdown compared with control knockdown cells (K and M). Knockdown of *p21* does not affect expression of p53 (H and J). (N) Quantification of *p21* mRNA levels by real-time PCR reveals a statistically significant increase in *p21* mRNA expression in TSA-treated control knockdown cells compared with DMSO-treated control knockdown cells (dark-gray bars) ($p < 0.05$) and no TSA-induced increase in *p21* expression in either *p21* (light- gray bars) or *p53* (medium-gray bars) knockdown cells.

(O) Model for interactions of HDAC1/2, ΔNp63, and p53 in embryonic epidermis. ΔNp63 and HDAC1/2 cooperate to directly repress expression of p16/INK4a and may also directly contribute to repression of p21. HDAC1/2 independently deacetylate p53, suppressing its ability to induce apoptosis and activate target genes, including p21. Data in (G) and (N) are represented as mean values; error bars represent SEM; asterisks indicate statistical significance; NS, not statistically significant.

See also Figure S5.

Consistent with HDAC1/2's functions in gene repression, we found that, whereas p63-activated basal layer genes were unaffected by *Hdac1/2* deletion, p63-suppressed genes, in particular *p16/Ink4a*, were markedly dysregulated. We further showed that HDAC1/2 bind the promoter regions of p63-repressed genes in undifferentiated keratinocytes and that histone acetylation is specifically increased in these regions following treatment with the HDAC inhibitor TSA. Together, these results indicate that HDAC1/2 directly mediate p63's repressive functions. Loss of the repressive functions of this key epidermal factor in *Hdac1/2* mutants may mask other less central roles of *Hdac1/2* at early stages of epidermal development, accounting for the striking similarity of *p63* null and *Hdac1/2* mutant phenotypes. HDAC1/2 are likely to play additional roles at subsequent embryonic stages and in adult skin and hair follicles. These may be revealed by inducible epidermal-specific deletion of *Hdac1/2* later in embryonic or postnatal life.

Interestingly, concomitant loss of *p16/Ink4a* partially rescues multiple defects in *p63* null epidermis, including progenitor cell proliferation, stratification, and hair follicle development (Su et al., 2009). Suppression of p16/INK4a expression is essential for self-renewal of multiple types of progenitor cells (Akala et al., 2008; Molofsky et al., 2006; Nishino et al., 2008). The partial rescue of stratification and hair follicle defects in *p63*$^{-/-}$

p16/Ink4a$^{-/-}$ double mutants suggests that this suppression is also essential for asymmetric cell division and cell fate decisions in embryonic epidermis. These data further suggest that the dramatic increase in p16/INK4a levels in *Hdac1/2* mutant epidermis contributes in a major way to the observed defects in epidermal development and hair follicle specification.

As mentioned above, the defects in *Hdac1/2* mutant epidermis were remarkably similar to those described for *p63* null skin. However, one difference was that p19/ARF is upregulated in *p63* mutants, but its levels were not substantially altered by *Hdac1/2* deletion. We discovered that HDAC1/2 are required to suppress hyper-acetylation of p53 in embryonic epidermis. Because p53 indirectly suppresses levels of p19/ARF (Kamijo et al., 1998; Stott et al., 1998), the competing effects of loss of p63-mediated repression and increased p53 activity may account for the relatively unchanged levels of p19/ARF in *Hdac1/2* compared with control epidermis. In addition to its possible contribution to regulation of p19/ARF, experiments in keratinocytes demonstrated that p53 is required for elevated p21 expression in response to HDAC inhibition. Thus, it is likely that hyper-acetylated p53 contributed to the observed upregulation of p21 and proliferative defects in *Hdac1/2* mutant epidermis.

Loss of *Hdac1/2* function in embryonic epidermis led to a gradual decrease in proliferation rates relative to controls and initially was not associated with increased apoptosis, suggesting that, as in *p63* null ectoderm (Senoo et al., 2007), failure of basal progenitor cell self-renewal contributes to the phenotype. Loss of either the epidermal histone methylase EZH2 or the DNA methyltransferase DNMT1 also results in defects in progenitor cell proliferation (Ezhkova et al., 2009; Sen et al., 2010). Epidermis lacking *Ezh2* displays increased p16/INK4a expression (Ezhkova et al., 2009), consistent with association of HDAC1/2 and EZH2 in the polycomb repressor complex PRC2 (van der Vlag and Otte, 1999). However, perhaps due to compensating activity of the related gene *Ezh1*, *Ezh2*-deficient epidermis displays a much less severe phenotype than that caused by loss of *p63* or *Hdac1/2* and is capable of stratifying and producing a functional barrier (Ezhkova et al., 2009).

Notably, unlike the effects of *Hdac1/2* deletion or absence of *p63*, loss of *Ezh2* or *DNMT1* is associated with premature differentiation of basal cells (Ezhkova et al., 2009; Sen et al., 2010). Thus, it is possible that *Hdac1/2* control progenitor cell differentiation in part through mechanisms distinct from those utilized by *Ezh2* and *DNMT1*. Alternatively, persistence of methylation marks in *Hdac1/2* mutants might block precocious activation of differentiation genes.

Unlike loss of EZH2 and DNMT1, lack of epidermal Mi-2β, which associates with HDAC1/2 in the NuRD complex, does not result in inappropriate activation of differentiation genes; instead, differentiation markers are expressed appropriately, and the epidermis stratifies normally. However, as in *Hdac1/2*-deficient epidermis, *Mi-2β* mutants display defects in hair follicle specification and a gradual decline in basal cell proliferation (Kashiwagi et al., 2007). These common phenotypes may reflect functions of the NuRD complex in hair follicle induction and progenitor cell self-renewal. Precisely how HDAC1/2 and p63 interact with Mi-2β, EZH1/2, DNMT1, and other chromatin modification factors to coordinate gene expression in the developing epidermis will be a fascinating area for further study.

In summary, our data reveal essential roles for *Hdac1/2* in proliferation, stratification, and cell fate decisions in the embryonic epidermis. Our results further suggest that *Hdac1/2* are required for mediating the repressive functions of p63 and for suppressing p53 activity. Because increased expression of HDACs in squamous cell carcinoma (SCC) predicts poor outcome, and HDACi can induce growth arrest in SCC lines as well as in normal keratinocytes, HDACs likely play key roles in SCC as well as in epidermal morphogenesis (Chang et al., 2009; Prystowsky et al., 2009; Saunders et al., 1999). Loss of *Hdac1/2* function results in derepression of cell cycle inhibitory and senescence factors in embryonic basal cells, suggesting specific targeting of these factors in undifferentiated epidermal tumors as a potentially powerful therapeutic tool. Determining the effects of *Hdac1/2* deletion in adult epidermis and in skin tumor models will be critically important in further testing the utility of this approach.

EXPERIMENTAL PROCEDURES

Generation of Mice

Mice carrying *Hdac1* and *Hdac2* floxed alleles (Montgomery et al., 2007) were crossed with transgenic *KRT14-Cre* line 43 mice (Andl et al., 2004). Genotyping was as described previously (Andl et al., 2004; Montgomery et al., 2007). All experiments were performed with approved animal protocols according to institutional guidelines established by the University of Pennsylvania IUCAC committee.

Histology, Immunostaining, and In Situ Hybridization

Histology, immunostaining, and whole mount in situ hybridization were carried out as described previously (Zhang et al., 2009). Details of the antibodies used are provided in Supplemental Experimental Procedures.

ChIP

ChIP was carried out according to the manufacturer's instructions using the EZ ChIP kit (Millipore) or the Magnify CHiP System (Invitrogen) and extracts of neonatal foreskin HEKs cultured in low-calcium medium (Li et al., 2005). For analyses of bound H3K9Ac, HEKs were cultured in 0 nM or 500 nM TSA in DMSO for 6 or 12 hr. Cells were fixed in 1% formaldehyde, lysed, and chromatin was sheared using a Bioruptor UCD-200 (Diagenode) to ~200 base pair fragments. Chromatin was immunoprecipitated with antibodies to HDAC1 (Abcam), HDAC2 (Abcam), H3K9Ac (Abcam), or Rabbit IgG (Millipore). Real-time PCR experiments coupled with ChIP utilized the StepOnePlus system and software (Applied Biosystems) and SYBR Green reagents (DNA Master). Changes were determined based on the $2^{-\Delta\ \Delta CT}$ method. See Table S1 for PCR primer sequences.

Assays for Proliferation and P53-AcK379 Expression in HEKs

Neonatal foreskin HEKs in low-calcium medium (Li et al., 2005) were plated at 20% confluency, treated with either 500 nM TSA in DMSO or DMSO alone for 24 hr, fixed with 4% paraformaldehyde, and immunostained with antibodies to PH3 (1:200) (Millipore) or P53-AcK379 (1:200) (Abcam).

shRNAmir Knockdown Experiments

Neonatal foreskin HEKs were transduced with retroviruses carrying shRNAmir nonsilencing, *p53*, or *p21* vectors (OpenBiosystems RHS4971, RHS4917-99781715, and RHS4917-99780100, respectively). Following puromycin selection, cells were treated with control (DMSO) or 50 nM TSA in DMSO for 24 hr, fixed, permeabilized, and immunostained with anti-PH3 (Millipore), p21 (Abcam), p53 (Abcam), or P53-AcK379 (Abcam). Each experimental condition was performed in duplicate, and the entire experiment was carried out twice. Detailed methods are provided in Supplemental Experimental Procedures.

Quantification of Transcript Levels by Real-Time PCR

Total RNA was purified using an RNeasy kit (QIAGEN), reverse-transcribed using First-Strand cDNA Synthesis Kit (GE, Amersham), and subjected to real-time PCR using the StepOnePlus system and software (Applied Biosystems) and SYBR Green reagents (DNA Master). See Supplemental Experimental Procedures for detailed methods and primer sequences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.devcel.2010.10.015.

ACKNOWLEDGMENTS

We thank L. Ash and T. Dentchev for histology, N. Sarikonda for technical assistance, and members of the S.E.M., E.E.M., and J.A.E. labs for helpful discussions. Research in S.E.M.'s lab is supported by RO1AR47709, RO1HD053829, RO1AR055241, and RC1DE020337. M.L. was supported by T32AR007465 and T32HD007516. J.A.E. and E.E.M. received support from UO1HL100405, and J.A.E. was supported by RO1HL071546.

Received: August 24, 2010
Revised: October 26, 2010
Accepted: October 26, 2010
Published online: November 18, 2010

REFERENCES

Akala, O.O., Park, I.K., Qian, D., Pihalja, M., Becker, M.W., and Clarke, M.F. (2008). Long-term haematopoietic reconstitution by Trp53−/−p16Ink4a−/−p19Arf−/− multipotent progenitors. Nature *453*, 228–232.

Andl, T., Ahn, K., Kairo, A., Chu, E.Y., Wine-Lee, L., Reddy, S.T., Croft, N.J., Cebra-Thomas, J.A., Metzger, D., Chambon, P., et al. (2004). Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. Development *131*, 2257–2268.

Blanpain, C., and Fuchs, E. (2007). p63: revving up epithelial stem-cell potential. Nat. Cell Biol. *9*, 731–733.

Brunmeir, R., Lagger, S., and Seiser, C. (2009). Histone deacetylase HDAC1/HDAC2-controlled embryonic development and cell differentiation. Int. J. Dev. Biol. *53*, 275–289.

Byrne, C., Tainsky, M., and Fuchs, E. (1994). Programming gene expression in developing epidermis. Development *120*, 2369–2383.

Candi, E., Terrinoni, A., Rufini, A., Chikh, A., Lena, A.M., Suzuki, Y., Sayan, B.S., Knight, R.A., and Melino, G. (2006). p63 is upstream of IKK alpha in epidermal development. J. Cell Sci. *119*, 4617–4622.

Chang, H.H., Chiang, C.P., Hung, H.C., Lin, C.Y., Deng, Y.T., and Kuo, M.Y. (2009). Histone deacetylase 2 expression predicts poorer prognosis in oral cancer patients. Oral Oncol. *45*, 610–614.

Chikh, A., Sayan, E., Thibaut, S., Lena, A.M., DiGiorgi, S., Bernard, B.A., Melino, G., and Candi, E. (2007). Expression of GATA-3 in epidermis and hair follicle: relationship to p63. Biochem. Biophys. Res. Commun. *361*, 1–6.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

Ezhkova, E., Pasolli, H.A., Parker, J.S., Stokes, N., Su, I.H., Hannon, G., Tarakhovsky, A., and Fuchs, E. (2009). Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell *136*, 1122–1135.

Frye, M., Fisher, A.G., and Watt, F.M. (2007). Epidermal stem cells are defined by global histone modifications that are altered by Myc-induced differentiation. PLoS ONE *2*, e763.

Haberland, M., Montgomery, R.L., and Olson, E.N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet. *10*, 32–42.

Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W., and Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol. Cell *1*, 3–11.

Higashitsuji, H., Masuda, T., Liu, Y., Itoh, K., and Fujita, J. (2007). Enhanced deacetylation of p53 by the anti-apoptotic protein HSCO in association with histone deacetylase 1. J. Biol. Chem. *282*, 13716–13725.

Indra, A.K., Dupe, V., Bornert, J.M., Messaddeq, N., Yaniv, M., Mark, M., Chambon, P., and Metzger, D. (2005). Temporally controlled targeted somatic mutagenesis in embryonic surface ectoderm and fetal epidermal keratinocytes unveils two distinct developmental functions of BRG1 in limb morphogenesis and skin barrier formation. Development *132*, 4533–4544.

Ingraham, C.R., Kinoshita, A., Kondo, S., Yang, B., Sajan, S., Trout, K.J., Malik, M.I., Dunnwald, M., Goudy, S.L., Lovett, M., et al. (2006). Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). Nat. Genet. *38*, 1335–1340.

Ito, A., Kawaguchi, Y., Lai, C.H., Kovacs, J.J., Higashimoto, Y., Appella, E., and Yao, T.P. (2002). MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. EMBO J. *21*, 6236–6245.

Jamora, C., DasGupta, R., Kocieniewski, P., and Fuchs, E. (2003). Links between signal transduction, transcription and adhesion in epithelial bud development. Nature *422*, 317–322.

Kamijo, T., Weber, J.D., Zambetti, G., Zindy, F., Roussel, M.F., and Sherr, C.J. (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. Proc. Natl. Acad. Sci. USA *95*, 8292–8297.

Kashiwagi, M., Morgan, B.A., and Georgopoulos, K. (2007). The chromatin remodeler Mi-2beta is required for establishment of the basal epidermis and normal differentiation of its progeny. Development *134*, 1571–1582.

Kim, J., Sif, S., Jones, B., Jackson, A., Koipally, J., Heller, E., Winandy, S., Viel, A., Sawyer, A., Ikeda, T., et al. (1999). Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. Immunity *10*, 345–355.

Koster, M.I. (2010). p63 in skin development and ectodermal dysplasias. J. Invest. Dermatol. *130*, 2352–2358.

Koster, M.I., Dai, D., Marinari, B., Sano, Y., Costanzo, A., Karin, M., and Roop, D.R. (2007). p63 induces key target genes required for epidermal morphogenesis. Proc. Natl. Acad. Sci. USA *104*, 3255–3260.

Laurikkala, J., Mikkola, M.L., James, M., Tummers, M., Mills, A.A., and Thesleff, I. (2006). p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. Development *133*, 1553–1563.

Lechler, T., and Fuchs, E. (2005). Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature *437*, 275–280.

Lefkimmiatis, K., Caratozzolo, M.F., Merlo, P., D'Erchia, A.M., Navarro, B., Levrero, M., Sbisa, E., and Tullo, A. (2009). p73 and p63 sustain cellular growth by transcriptional activation of cell cycle progression genes. Cancer Res. *69*, 8563–8571.

Li, W., Marshall, C., Mei, L., Dzubow, L., Schmults, C., Dans, M., and Seykora, J. (2005). Srcasm modulates EGF and Src-kinase signaling in keratinocytes. J. Biol. Chem. *280*, 6036–6046.

Liu, F., Thirumangalathu, S., Gallant, N.M., Yang, S.H., Stoick-Cooper, C.L., Reddy, S.T., Andl, T., Taketo, M.M., Dlugosz, A.A., Moon, R.T., et al. (2007). Wnt-beta-catenin signaling initiates taste papilla development. Nat. Genet. *39*, 106–112.

Liu, F., Chu, E.Y., Watt, B., Zhang, Y., Gallant, N.M., Andl, T., Yang, S.H., Lu, M.M., Piccolo, S., Schmidt-Ullrich, R., et al. (2008). Wnt/beta-catenin signaling directs multiple stages of tooth morphogenesis. Dev. Biol. *313*, 210–224.

Lopardo, T., Lo Iacono, N., Marinari, B., Giustizieri, M.L., Cyr, D.G., Merlo, G., Crosti, F., Costanzo, A., and Guerrini, L. (2008). Claudin-1 is a p63 target gene with a crucial role in epithelial development. PLoS ONE *3*, e2715.

Marinari, B., Ballaro, C., Koster, M.I., Giustizieri, M.L., Moretti, F., Crosti, F., Papoutsaki, M., Karin, M., Alema, S., Chimenti, S., et al. (2009). IKKalpha is a p63 transcriptional target involved in the pathogenesis of ectodermal dysplasias. J. Invest. Dermatol. *129*, 60–69.

McDade, S.S., and McCance, D.J. (2010). The role of p63 in epidermal morphogenesis and neoplasia. Biochem. Soc. Trans. *38*, 223–228.

Mikkola, M.L., and Millar, S.E. (2006). The mammary bud as a skin appendage: unique and shared aspects of development. J. Mammary Gland Biol. Neoplasia *11*, 187–203.

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature *398*, 708–713.

Molofsky, A.V., Slutsky, S.G., Joseph, N.M., He, S., Pardal, R., Krishnamurthy, J., Sharpless, N.E., and Morrison, S.J. (2006). Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature *443*, 448–452.

Montgomery, R.L., Davis, C.A., Potthoff, M.J., Haberland, M., Fielitz, J., Qi, X., Hill, J.A., Richardson, J.A., and Olson, E.N. (2007). Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. Genes Dev. *21*, 1790–1802.

Nishino, J., Kim, I., Chada, K., and Morrison, S.J. (2008). Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression. Cell *135*, 227–239.

Prystowsky, M.B., Adomako, A., Smith, R.V., Kawachi, N., McKimpson, W., Atadja, P., Chen, Q., Schlecht, N.F., Parish, J.L., Childs, G., et al. (2009). The histone deacetylase inhibitor LBH589 inhibits expression of mitotic genes causing G2/M arrest and cell death in head and neck squamous cell carcinoma cell lines. J. Pathol. *218*, 467–477.

Romano, R.A., Ortt, K., Birkaya, B., Smalley, K., and Sinha, S. (2009). An active role of the DeltaN isoform of p63 in regulating basal keratin genes K5 and K14 and directing epidermal cell fate. PLoS ONE *4*, e5623.

Rosemary Siafakas, A., and Richardson, D.R. (2009). Growth arrest and DNA damage-45 alpha (GADD45alpha). Int. J. Biochem. Cell Biol. *41*, 986–989.

Ruthenburg, A.J., Li, H., Patel, D.J., and Allis, C.D. (2007). Multivalent engagement of chromatin modifications by linked binding modules. Nat. Rev. Mol. Cell Biol. *8*, 983–994.

Saifudeen, Z., Marks, J., Du, H., and El-Dahr, S.S. (2002). Spatial repression of PCNA by p53 during kidney development. Am. J. Physiol. Renal Physiol. *283*, F727–F733.

Saunders, N., Dicker, A., Popa, C., Jones, S., and Dahler, A. (1999). Histone deacetylase inhibitors as potential anti-skin cancer agents. Cancer Res. *59*, 399–404.

Sen, G.L., Reuter, J.A., Webster, D.E., Zhu, L., and Khavari, P.A. (2010). DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature *463*, 563–567.

Senoo, M., Pinto, F., Crum, C.P., and McKeon, F. (2007). p63 Is essential for the proliferative potential of stem cells in stratified epithelia. Cell *129*, 523–536.

Stott, F.J., Bates, S., James, M.C., McConnell, B.B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K.H., et al. (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J. *17*, 5001–5014.

Su, X., Cho, M.S., Gi, Y.J., Ayanga, B.A., Sherr, C.J., and Flores, E.R. (2009). Rescue of key features of the p63-null epithelial phenotype by inactivation of Ink4a and Arf. EMBO J. *28*, 1904–1915.

Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008). Acetylation is indispensable for p53 activation. Cell *133*, 612–626.

Truong, A.B., and Khavari, P.A. (2007). Control of keratinocyte proliferation and differentiation by p63. Cell Cycle *6*, 295–299.

Truong, A.B., Kretz, M., Ridky, T.W., Kimmel, R., and Khavari, P.A. (2006). p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev. *20*, 3185–3197.

van der Vlag, J., and Otte, A.P. (1999). Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat. Genet. *23*, 474–478.

Westfall, M.D., Mays, D.J., Sniezek, J.C., and Pietenpol, J.A. (2003). The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. Mol. Cell. Biol. *23*, 2264–2276.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C., et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature *398*, 714–718.

Zhang, Y., Tomann, P., Andl, T., Gallant, N.M., Huelsken, J., Jerchow, B., Birchmeier, W., Paus, R., Piccolo, S., Mikkola, M.L., et al. (2009). Reciprocal requirements for EDA/EDAR/NF-kappaB and Wnt/beta-catenin signaling pathways in hair follicle induction. Dev. Cell *17*, 49–61.